Docket No. 55046 (70207)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S):	T. C. Walsh, et al.	EXAMINER:	K. M. Kerr
SERIAL NO.:	10/017,324	GROUP:	1652
FILED:	December 15, 2001		
FOR:	METHODS FOR PREPARATION OF MACROCYCLIC MOLECULES AND MACROCYCLIC MOLECULES PREPARED THEREBY		

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SIR:

DECLARATION UNDER 37 CFR 1.131

The undersigned declare as follows:

1. We are co-inventors of the above-identified application assigned to the President and Fellows of Harvard College.

2. Prior to September, 2000, we had reduced to practice reactions preparing macrocyclic molecules by contacting a excised thioesterase (TE) domain with a substrate that contained a nucleophile and an activated acyl residue.

3. Prior to September, 2000, we had reduced to practice macrocyclization substrates for use in preparing macrocyclic molecules that contained a nucleophile and an activated thioester group.

4. Prior to September, 2000, such macrocyclization substrates had been contacted in an aqueous media with a purified excised TE domain under conditions conducive to macrocycle formation. As evidence thereof, attached as Exhibit 1 are selected portions of a disclosure of the subject matter of the above-identified application. The disclosure attached as Exhibit 1 was generated, and actual experimental work disclosed therein was performed, prior to September, 2000. Portions of the disclosure attached as Exhibit 1, including specific dates, have been removed.

5. We hereby further declare that all statements made herein are of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Date: _____ _____
John W. Trauger

Date: _____ _____
Rahul M. Kohli

Date: _____ _____
Henning D. Mootz

Date: _____ _____
Mohamed A. Marahiel

Date: _____ _____
Christopher T. Walsh

Date: _____ _____
Dirk Schwarzer

Date: 06/09/2004 _____
Michael D. Burkart

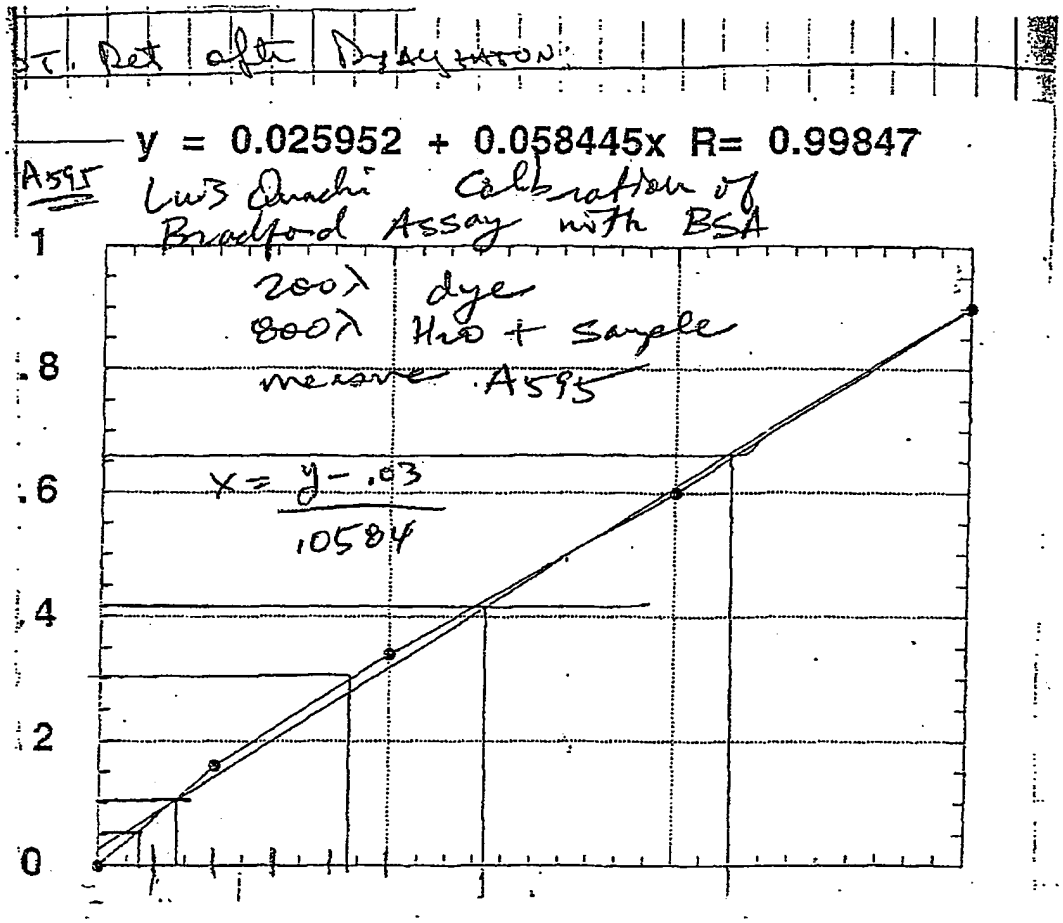
BOS2_442602.1

Expression ① pQE60-TE in SG13009(pREP4) (Amp^R)
② pA4-His6 in BL21(DE3) (Amp^R)
③ pA6-His6 in BL21(DE3), pLysS (Amp^R/cm^R)

— overnight preculture 30°C
— inoculate 15 mL/L LB + antibiotic(s)
— shake:

Time	(1×1L) A4	(1×1L) A6	(2×1L) TE	
2 hr	.049	.22	.36	34°C → RT
2.5 hr.	.058	.36	(.54) induce w/ 400× 1M IPTG	26°C
3 hr.	.069	(.51)		26°C
	finished	induce w/ 200× 1M IPTG		
7 hr, 45 min	X	2.0	2.0	26°C

— Harvest, resuspend low imidazole 1× lys3 buffer (2 mM imidazole)



pQE-TE purification

from p.16 - expressed in "56 flow" cells (Qiagen 13009 [PREP4])
- French press (2x) spin 15,000 rpm/45 min.
- Shake supernatant w/ 4 mL Ni-NTA (50% slurry, washed 1x with 40 mL buffer) at 4°C for 2.5 hours
- Pellet resin; ~~5,000~~ 3000 rpm/5 min
- Transfer to small column
- Wash at 1 mL/min for 60 min. with 20mM imidazole, 300mM NaCl, 20 mM Tris, pH 8.
- Then elute with 20mM → 250 mM imidazole gradient in 60 min. The product eluted at (very approx) 70 mM imidazole.
- Protein-containing fractions identified by Bradford assay (4λ fraction + 200λ 1:4 dye), spot into early and late (6-7 and 8-9, respectively) fractions, and dialyzed overnight at 4°C vs. 1

50 mM Tris-HCl, pH 8
100 mM NaCl
10 mM $MgCl_2$
1 mM EDTA
10% glycerol

ε_{280} estimation:
1.7 mM ⇔ $A_{280} = 0.094$
$A = \varepsilon c l$
$\varepsilon = \frac{A}{c} = \frac{.094}{1.7 \times 10^{-3} M \cdot 1cm}$
$= 55,294 \approx 55,000$

- Quickfreeze in LN_2 and ~~freeze~~ store at -80°C.
- A_{280} of 1:25 dilutions:

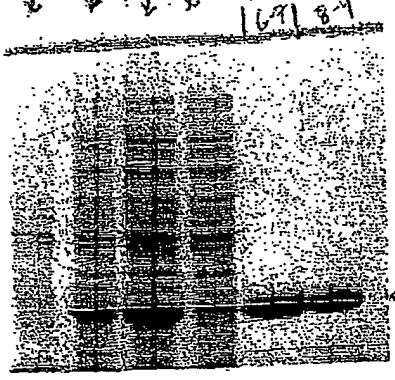
6-7	8-9
0.094	0.073

- Bradford Assay (fraction 6-7 only)

	A_{595}
200λ dye + 800λ H_2O + SAMPLE ∅ (blank)	.490
1λ	.603 .11
2λ	.712 .21
4λ	.857 .37

conc (vs. BSA) ≈ 1.4 μg/mL × 1000 = 1.4 mg/mL
3.1 μg/mL × 500 = 1.5 mg/mL

1.5 mg/mL



mw ≈ 35 kD

$\frac{1.5 mg}{10000 mL} \times \frac{35,000 mg}{mmol} = .00004 \frac{mmol}{mL}$

$\frac{0.04 mM}{42.9 cm}$

Synthesis of tyrocidine linear peptide

H2N - D-Phe - Pro - Phe - D-Phe - Asn - Gln - Tyr - Val - Orn - Leu - COOH

Notebook file is "TLP1.NBK".

Target Peptide: length = 10, MW = 1288.521

NH2-END-Phe-SPC-Pro-SPC- DC-Phe-SPC-Phe-SPC-Asn-SPC-Gln-SPC-Tyr-SPC- DC-Val-SPC- DC-Orn-SPC-Leu-COOH

Support substitution = 0.750 meq/g
Support quantity = 0.400 g
Excess amino acid = 3.000 x
] Synthesis on 2-Cl-Trityl resin (acid-sensitive linker).

Peptide Quantity = 0.300 mMoles
Theoretical Yield = 0.387 g

0.9 mmol × 265.3 mg/mmol = 239 mg

Starting Support: FMOC-Leu-Peptide-Acid

2 = HOBt only for Pfp esters
3 = HOBt activation w/ DIPCDI

Added 138 mg HOBt => 0.9 mmol (MW = 153.1) to each vial first.

Cycle	AA	Proto	Time	Derivative		Grams	mL	Vial
22)	SPC	N	00:07:50	System Preparation		0.000		1
21)	Orn	B3*	00:50:10	Fmoc-L-Orn(Boc)-OH	Orn	~~0.409~~	3.4	2
20)	DC	H3*	00:35:10	Double Couple		~~0.409~~	3.4	3
19)	SPC	J	00:09:15	N-Acetylimidazole		0.099	3.1	4
18)	Val	B2	00:50:10	Fmoc-L-Val-OPfp	Val	~~0.455~~	3.5	5
17)	DC	H2	00:35:10	Double Couple		~~0.455~~	3.5	6
16)	SPC	J	00:09:15	N-Acetylimidazole		0.099	3.1	7
15)	Tyr	B3*	00:50:10	Fmoc-L-Tyr(tBu)-OH	Tyr	~~0.412~~	3.4	8
14)	SPC	J	00:09:15	N-Acetylimidazole		0.099	3.1	9
13)	Gln	B2	00:50:10	Fmoc-L-Gln(Trt)-OPfp	Gln	~~0.699~~	3.7	10
12)	SPC	J	00:09:15	N-Acetylimidazole		0.099	3.1	11
11)	Asn	B2	00:50:10	Fmoc-L-Asn(Trt)-OPfp	Asn	~~0.686~~	3.7	12
10)	SPC	J	00:09:15	N-Acetylimidazole		0.099	3.1	13
9)	Phe	B3*	00:50:10	Fmoc-D-Phe-OH	D-Phe	~~0.349~~	3.3	14
8)	SPC	J	00:09:15	N-Acetylimidazole		0.099	3.1	15
7)	Phe	B2	00:50:10	Fmoc-L-Phe-OPfp		~~0.498~~	3.5	16
6)	DC	H2	00:35:10	Double Couple	L-Phe	~~0.498~~	3.5	17
5)	SPC	J	00:09:15	N-Acetylimidazole		0.099	3.1	18
4)	Pro	B2	00:50:10	Fmoc-L-Pro-OPfp	Pro	~~0.453~~	3.5	19
3)	SPC	J	00:09:15	N-Acetylimidazole		0.099	3.1	20
2)	Phe	B3*	00:50:10	Boc-D-Phe-OH MW 265.3	0.239 D-Phe	~~0.349~~	3.3	21
1)	END	G	00:07:15	Final Cycle		0.000		22

Minimum loop size = 10 mL
Installed loop size = 10 mL

Estimated time required for synthesis completion: 10:46:05

Estimated Reagent consumption and requirements for synthesis completion:

	consumption	required
MF Main Wash	770 mL	870 mL

Deblock	229 mL	329 mL
Wash 2	31 mL	131 mL
Aux Wash	61 mL	161 mL
Syringe 2	34 mL	46 mL
Syringe 3	24 mL	36 mL
AAM Wash	200 mL	300 mL
Synth Waste	819 mL	
SP1 Waste	56 mL	
AAM Waste	477 mL	

Quantity Chemistry

- After synthesis, blow out resin with N_2, dry on lyophilizer for a while (with 5 hrs started at ~1 AM).
- I will try the acetic acid cleavage method because it looks easiest (1 step). Treat resin in scint vial w/ Teflon top with AcOH/TFE/DCM (2:2:6) for 2 hours at R.T. (Novabiochem p. 555).

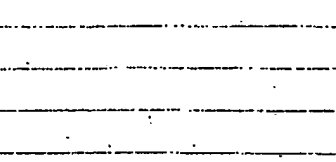
100 mL cleavage mix:	20 mL AcOH
	20 mL TFE
	60 mL DCM

Filter, check filtrate by TLC
Wash 3x with cleavage mix, monitor by TLC — trace (decreasing amts) in all washes.
(Tried second 2 hour cleavage, see no additional material ⇒ cleavage done in 2 hr).
Add 15 volumes hexane and rotovap dry, add 50 mL hexane and dry. Transfer to 25 mL flask w/ CH$_2$Cl$_2$, dry mostly, add hexane and dry to white solid. Single spot by TLC (18% MeOH/CH$_2$Cl$_2$) (visualize by UV). Tare: 33.5757g
Yield: 462 mg MW = (protected) 1029.5 = 0.45 mmol (76% not bad!)

Boc-D-Phe-Pro-L-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-COOH
Trt Trt tBu Boc

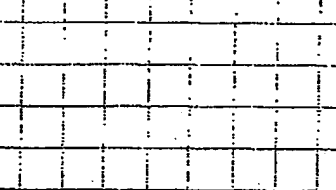
Thioester formation

(Prep2) Boc-DPhe-Pro-Phe-D-Phe-Asn(Trt)-Gln(Trt)-Tyr(tBu)-Val-Orn(Boc)-Leu-Cos



Boc-D-Phe-Pro-Phe-D-Phe-Asn(Trt)-Gln(Trt)-Tyr(tBu)-Val-Orn(Boc)-Leu-Cys

(III-116A)



Bzl-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-Cys

(III-116B)

TLP

tyrosine linear peptide

	Amt.	Mmol	Eq	MW	d
Prep2	157 mg	0.077	1	2029.5	
N-Ac-cysteine	~~8.5~~ 10 µL		1.1	119.2	1.12
DCC	16 mg		1	206.3	
HOBt	12 mg		1	153.1	
K_2CO_3	5 mg				
THF	0.5 mL				

→ Prep2 ←
~~cleaved using 1:1:3 TFE/AcOH/DCM mix (~5 mL)~~
for 3 hours at RT. Filter, wash 2x 2 mL cleavage mix.
→ Thioester formation [Dry repeatedly (4x) from CH_2Cl_2/hexane to remove AcOH. Dry high-]
work-up; Filter, ~~concentrate~~ dry down extract w/ 10% $NaHCO_3$
to remove HOBt (extract into CH_2Cl_2 or EtOAc). Then
concentrate and check the purity.
Yield: ~~colorless~~ (white) solid: 313 mg

[4x DCC/HOBt mix: ~~50~~ 64 mg DCC, } in 1 mL THF
48 mg HOBt } add 0.25 mL/rxn

[~~Peptide~~: 313 mg dissolved in 0.5 mL THF
add 0.25 mL/rxn

[Take 8 μL sample = 5 mg]
Sol'n becomes cloudy w/ white DCU ppt in < 5 minutes
Vigorous stirring. Add N-Ac-cysteamine after 5 min
with DCC/HOBt. Stir 3½ hours at RT.
~~Filter to remove DCU~~

(margin: give MS for selected peptide. [M+Na] 0.50.93 calc'd for [M+Na] = 51.0)

Deprotect: use ~~80%~~ TFA / CH_2Cl_2 / ~~10%~~ 5% N-Acetylcysteamine (scavenger)
Deprotect for 2 hours at RT. Solution turned
bright yellow upon adding deprotect mix. use small
volume (~2 mL). Then add ~50 mL cold ether
dropwise, then store at 4°C for 3 hours. Fine
white powder. Recover by centrifugation, wash
3x with cold ether. Then dissolve in
20% CH_3CN / 0.1% TFA and purify by prep.
HPLC (Waters, monitor at 240 nm) 3 injections
efficient removal of the free acid. Lyophilize
Gradient: [to dryness. Then take up in H_2O (1 mL per
mg of peptide and lyophilize).

(margin: we get 40 mg per 0.3 mmol thioester synthesis)

Characterization: (III-116B)
Yield: 20 mg [M+H] colorless powder ← ~20% overall - ok.
✓ MALDI-TOF MS: ^measured exact mass = 1389.74 (calc'd = 1389.7)
✓ HPLC: single peak; trace of free acid.
✓ λmax: ~220 nm (ε rel = 10), ~280 nm (ε rel = 1)

looks good!

ε_{280} (calculated) = 1280 $M^{-1} cm^{-1}$ (ProtParam tool), (www.expasy)

2nd attempt to cyclize TLPI-SMAC (EM-1168)

Previously I found that Tris·HCl at pH 8 causes precipitation of TLPI-SMAC. This did not occur with HEPES·NaOH, pH 7, even after adding 140 mM NaCl. Conc of TLPI-SMAC in this trial was 7 mM.

Step 1: Dialyze QE60-TE vs. the following buffer overnight at 4°C:

assume [enzyme] = 40 μM

25 mM HEPES·NaOH, pH 7.0
50 mM NaCl
10% glycerol

QE60 prep is labeled "TE89"

→ Note: ± dialyzed material called "fract. 8-9" (see R17).

Step 2: Dissolve peptide in buffer/NaCl (first dissolve in water, then add buffer, then NaCl; (47 mM stock),

1.8 mg + 140 λ H2O
7.5 λ 0.5 M HEPES, pH 7
1.9 λ 4 M NaCl.

Step 3: Set rxns:

① no peptide control (enzyme only)
② no enzyme control (peptide only)
③ "Time 0" 20 λ enzyme + 20 λ peptide

Final [TLPI-SMAC] ≈ 3.5 mM
Final [enzyme] ≈ 20 μM

ppt. forms, remains upon dilution with additional 60 λ H2O. [NaCl] ≈ 50 mM

ppt. NOT DUE TO:
- glycerol in enzyme
- TFA anion in ~~buff~~ peptide prep

"Time 1" ④ To 20λ peptide, add 60λ H_2O, then 10λ enzyme. cloudy solution, dark so transfer before (Time 0 sample).

[peptide] = 1.6 mM
[enzyme] = 4 μM
[NaCl] = 17 mM

"Time 2" ⑤ ~~B~~ 20λ 7mM TLP1:SMAC
2λ 4M NaCl
7λ 0.5 M HEPES,
108λ H_2O
3.5λ TE
700λ

[peptide] = 1 mM
[enzyme] = 1 μM
[NaCl] = 50 mM
slightly cloudy solution

"Time 3" ⑥ Same as "Time 2" but no salt added.

[peptide] = 1 mM
[enzyme] = 1 μM
[NaCl] = 8 mM
[~~PBS~~ HEPES] = 25 mM
pH = 7
slightly cloudy ..., but less so than Time 2
soln might get cloudier at 37°C, -
it seemed to in w 5 min so
took all rxns out to RT.

LOW SALT IS GOOD — ROOM TEMP MAY BE PREFERABLE.
Start at noon

MAKE PEPTIDE SOLUTION IN H_2O, allowing you to reach lower [NaCl].

Ideal reaction conditions (Ethik): no NaCl, except a little bit from the enzyme prep.

Condition A: 10% ~~[illegible]~~ 7 mM peptide in H_2O
3.5% ~~[illegible]~~ 0.5 M HEPES, pH 7
55% ~~[illegible]~~ H_2O
1.0% ~~[illegible]~~ TE 89
70 ~~[illegible]~~%

[HEPES] = 25 mM
[peptide-SMAC] = 1 mM
[TE] = 1 μM
[NaCl] = 1.3 mM

Peptide-Stock: 0.75 mM TLPI-SMAC in 58 μL

if soluble, try to increase enzyme concentration OR peptide-SMAC concentration.

SOLUBILITY CHECK: TLPI-SMAC is readily soluble in n-butanol ⇒ this is a good solvent for extraction: extract mixt w/ n-BuOH then concentrate to dryness. Resuspend residue in 20% CH_3CN / 0.1% TFA/water. Run HPLC sample. This will remove the enzyme.

nBuOH bp = 117°C (BuNH$_2$ bp = 153°C).
Rotovap from glass vials.

Quench all reactions (this page and pp 120-121) by freezing at −80°C.

Isolate peptides by extraction with butanol (2 x one volume). Then concentrate on rotovap onto a glass vial using high vaccuum. Resuspend in ~~[illegible]~~ 20% CH_3CN / 0.1% TFA in H_2O.

Run HPLC. Inject 70% sample, Gradient 0 → 100% CH_3CN in 0.1% TFA/water.

Rxn time: ~20 hours at Room Temp

Rxn A: condition A at 1pt

Rxn B: control; no enzyme

start ~1 PM at RT.
Quench (freeze -20°C) at PM.

1 PM → 10 AM = 21 hours vs.
5 hours (factor of 4).

Rxn C: control. no peptide.

TE domain control; rxn set normally w/ no peptide; diluted to 140x with water. Inject 20x HPLC.

Rxn A: see SM consumed, hydrolysis + new peak ("cycle") (+ just TE)

Rxn B: see some hydrolysis of SM (~30%) (no enzyme)

Rxn C: see nothing (no peptide)

SM: starting material purity is very good!

TE: injected TE domain on HPLC.

new peak collected and submitted for MS

New peak "cycle" on p. 124 collected off of HPLC column
MS results:
① MALDI-TOF (DHB) calc'd: 1270.65
observed: 1270.68 ✓

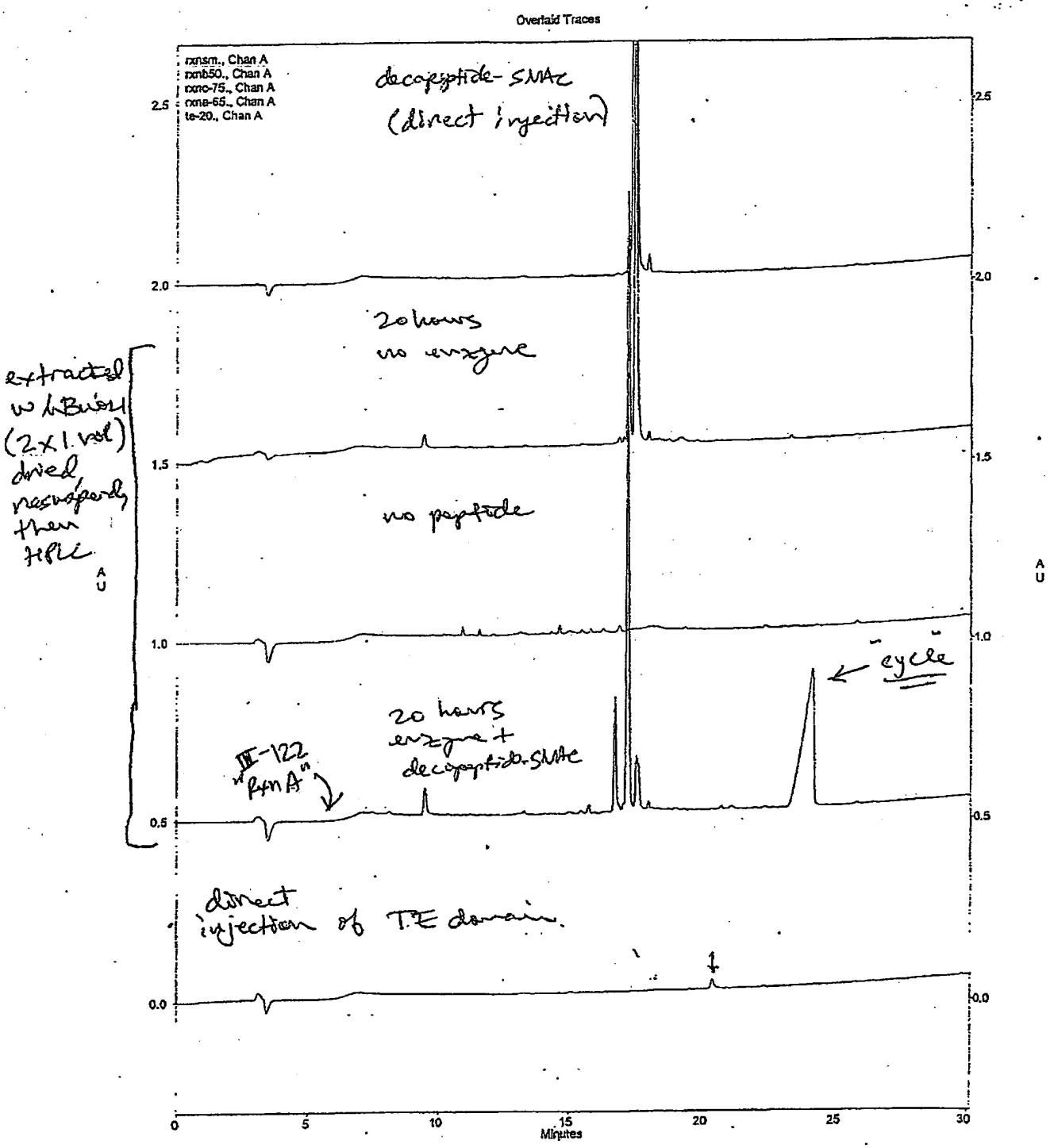
See data on pp 130-131.

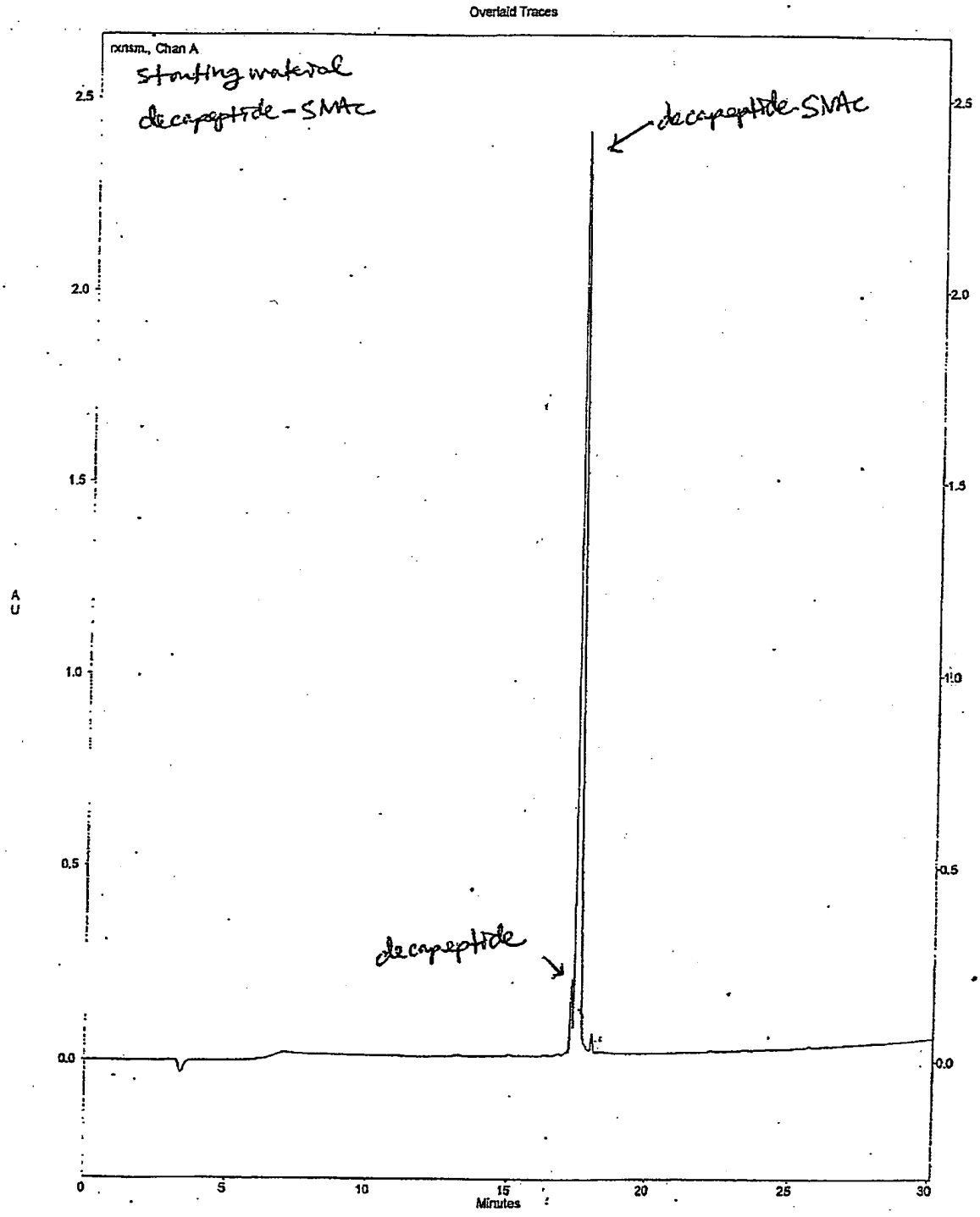
John Frazier

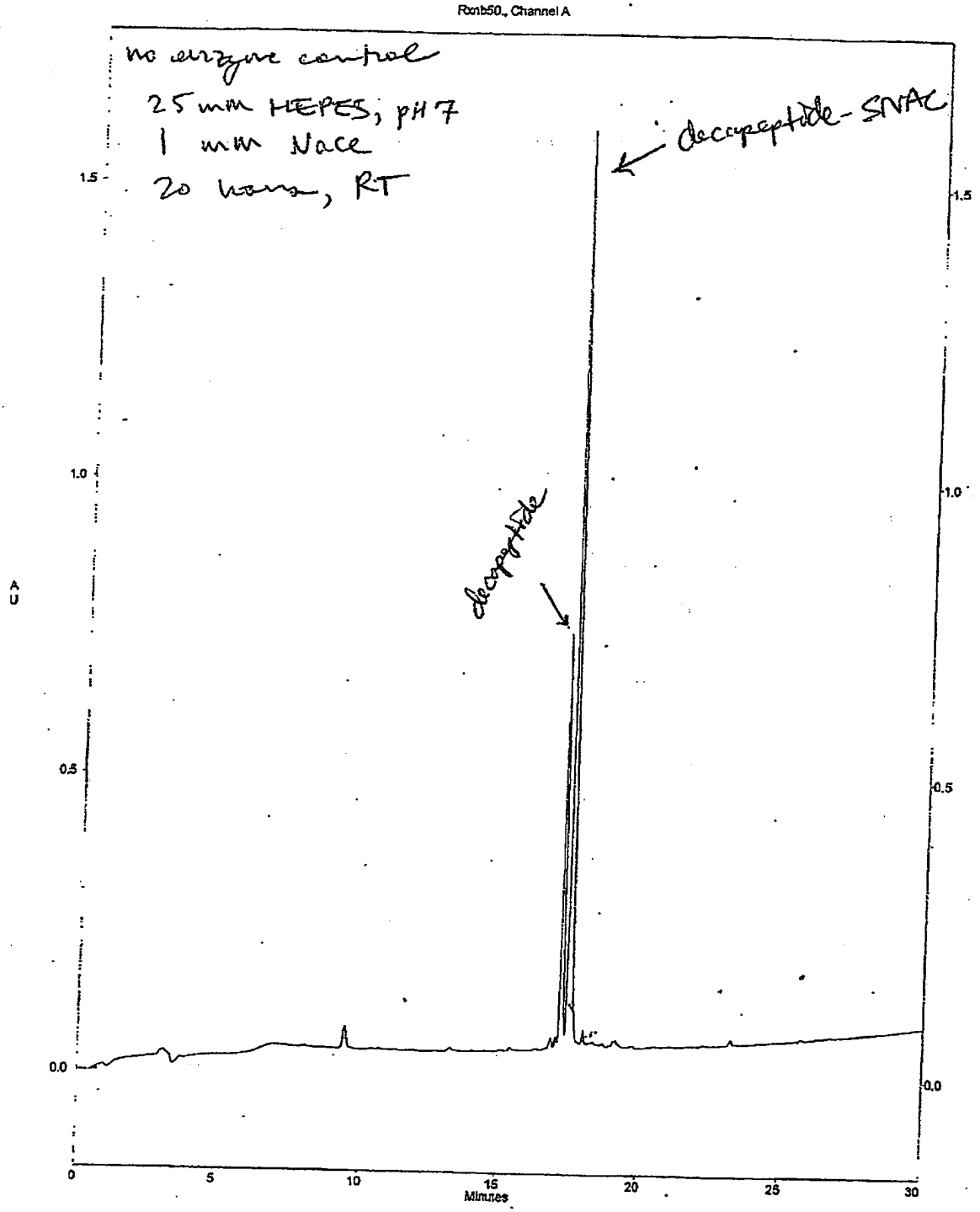
1 0 1 slow Trace

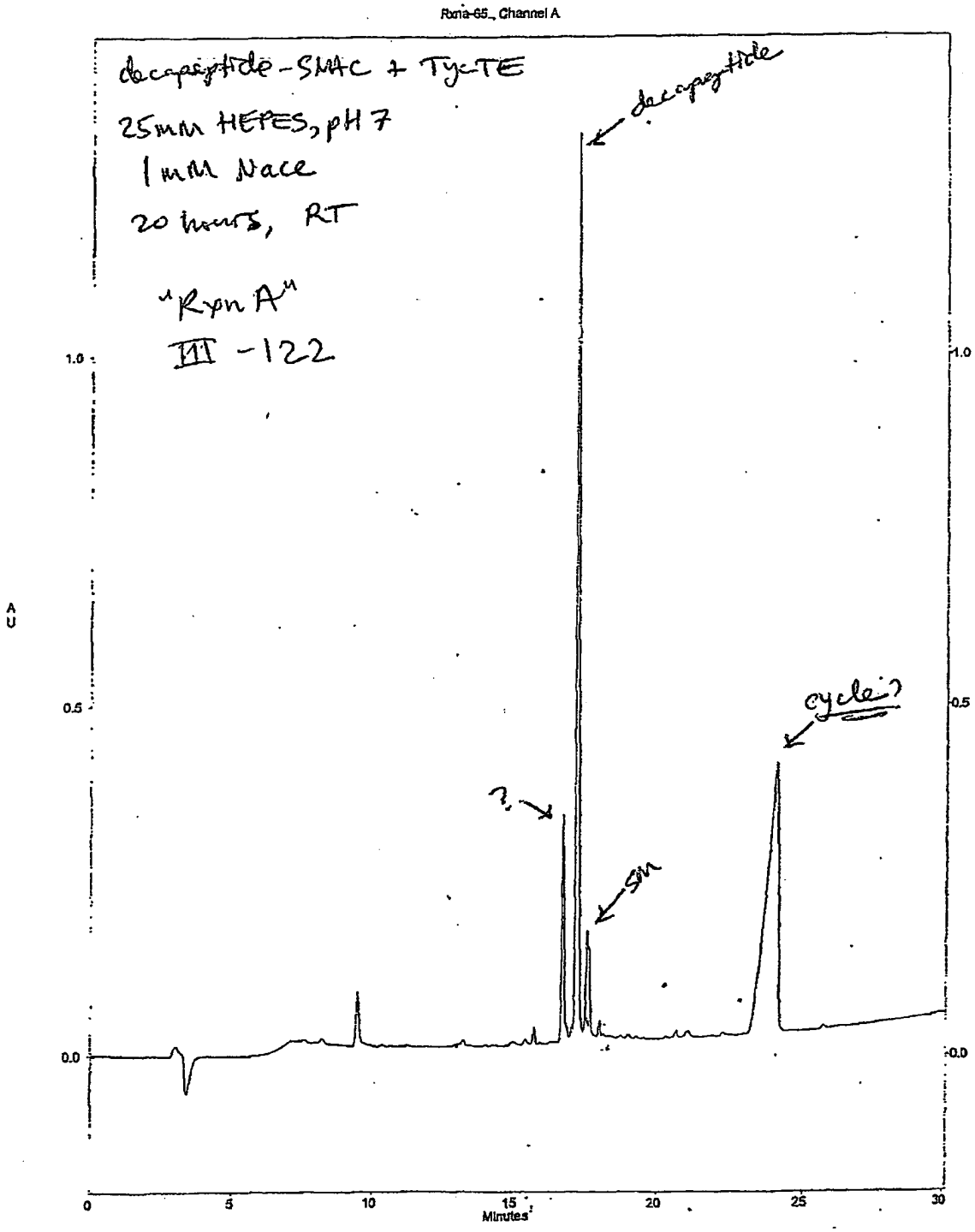
John Trace 0 → 100% CH_3CN (0.1%TFA) in H_2O (0.1% TFA) in 30 minutes.

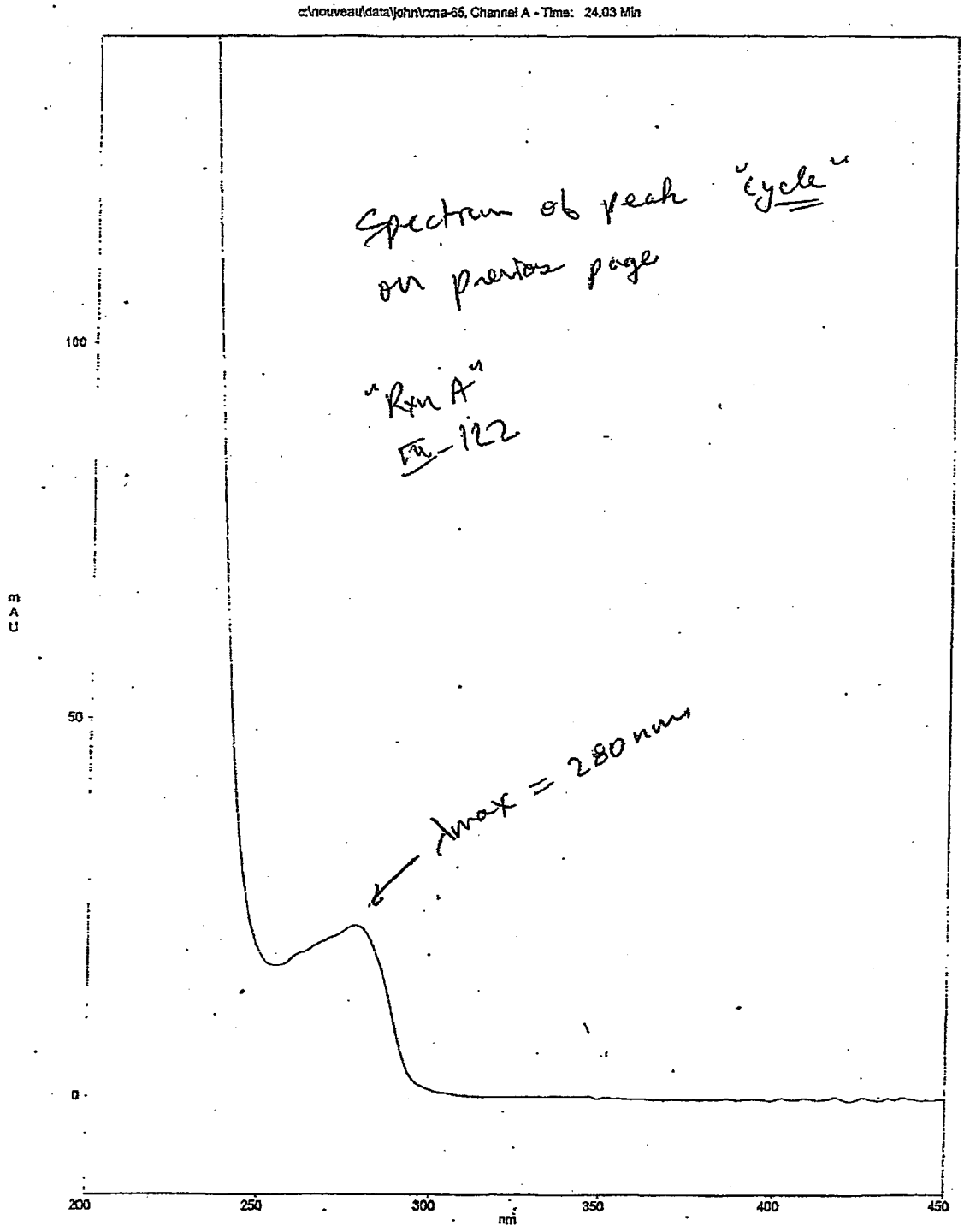
JWT-III-124




Overlaid Traces
rxnsm., Chan A
starting material
decapeptide-SNAc
decapeptide-SNAc
decapeptide
AU
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
Minutes


Rcnb50_Channel A
no enzyme control
25 mM HEPES; pH 7
1 mM NaCl
20 hours, RT
decapeptide-SNAC
decapeptide
AU
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
Minutes

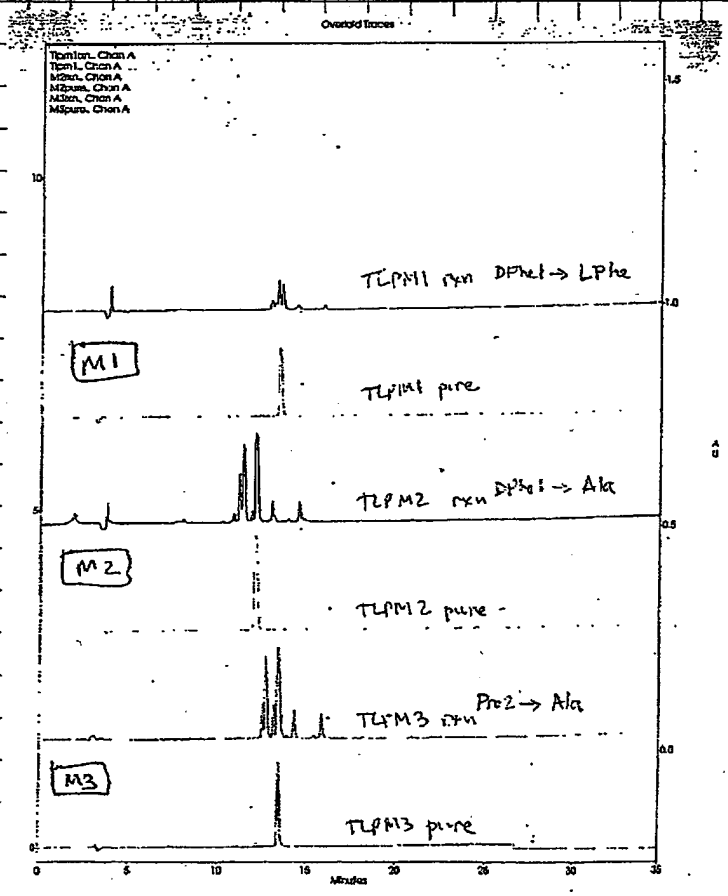

Rona-65_ Channel A
decapeptide-SMAC + Tyr-TE
25mM HEPES, pH 7
1mM NaCl
20 hours, RT
"Ryn A"
III -122
decapeptide
cycle?
?
SM
A
U
1.0
0.5
0.0
0
5
10
15
20
25
30
Minutes

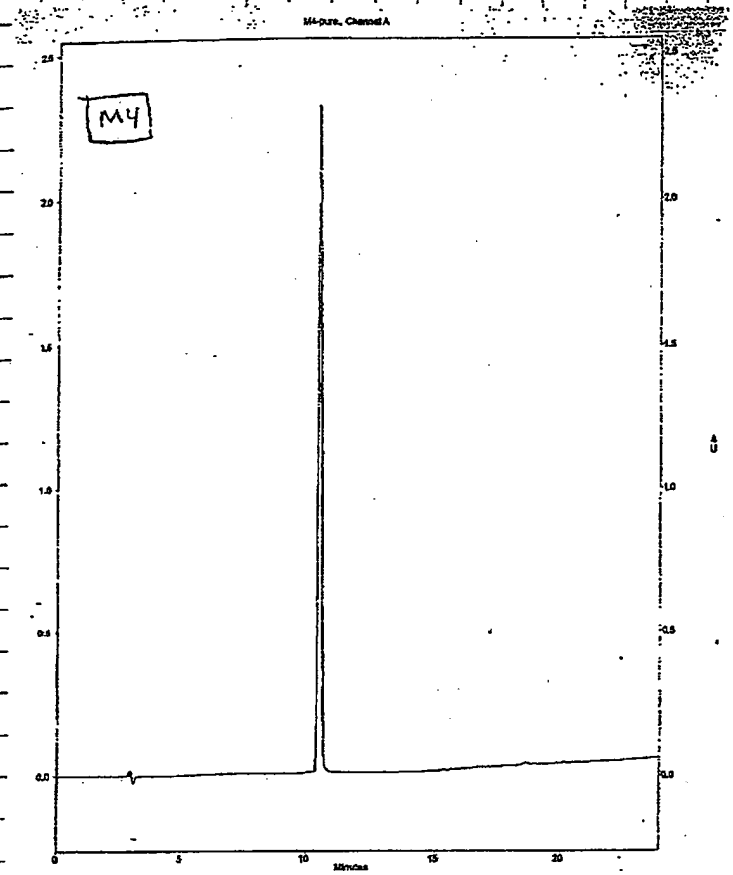

c:\nouveau\data\john\xna-65, Channel A - Time: 24.03 Min
Spectrum of peak "cycle" on previous page
"Run A"
Fr. 122
100
50
0
mAU
λmax = 280 nm
200
250
300
350
400
450
nm

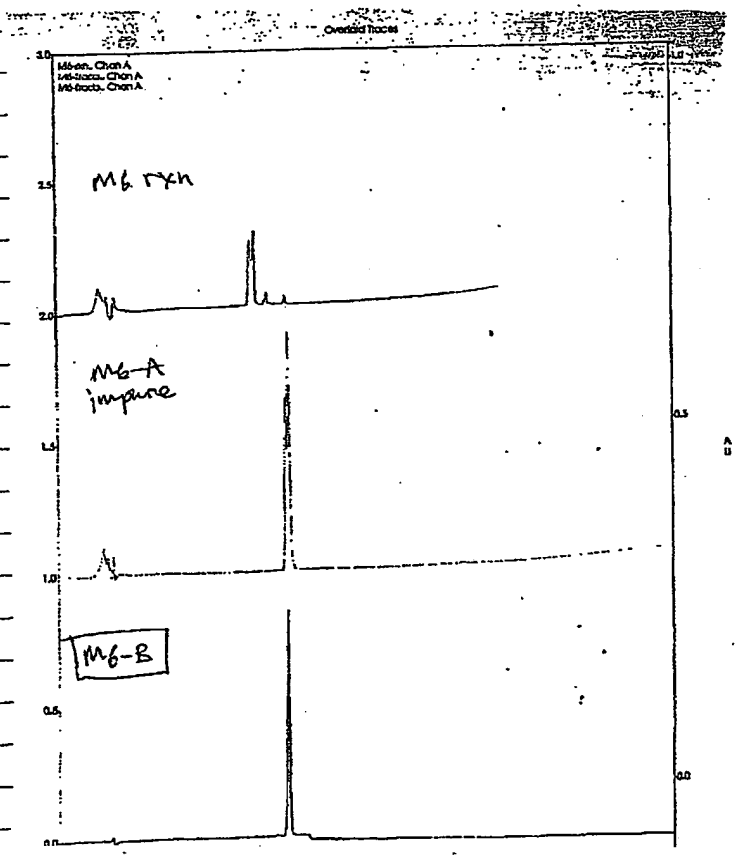
Synthesis of peptide-SNACs

Name	Sequence	MW (TFA salt)	M+H (calc'd)	M+H (observe
TLP-SNAC	DPhe-Pro-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-Leu	1617.7	1389.7	1389.7
TLP-M1-SNAC	**Phe**-Pro-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-Leu	1617.7	1389.7	1390.1
TLP-M2-SNAC	**DAla**-Pro-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-Leu	1541.6	1313.7	1313.9
TLP-M3-SNAC	DPhe-**Ala**-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-Leu	1591.7	1363.7	1363.9
TLP-M4-SNAC	DPhe-Pro-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-**Ala**	1575.6	1461.6	
TLP-M5-SNAC	DPhe-Pro-Phe-Asn-Gln-Tyr-Val-Orn-Leu	1470.5	1242.6	
TLP-M6-SNAC	DPhe-Pro-Phe-DPhe-Asn-**Ala**-Gln-Tyr-Val-Orn-Leu	1688.8	1460.7	
3LP5-SNAC	DPhe-Pro-Val-Orn-Leu	918.0	690.4	690.6
3LP10-SNAC	DPhe-Pro-Val-Orn-Leu-DPhe-Pro-Val-Orn-Leu	1602.7	1260.8	

- All peptide-SNACs purified by reverse-phase C18 chromatography (HPLC) using 0.1% TFA/acetonitrile.
- Characterize by MALDI-TOF MS (DFCI)




M4-pure, Channel A
M4
AU
Minutes


Overlaid Traces
M6-en, Chan A
M6-trace, Chan A
M6-trace, Chan A
M6 rxn
M6-A impure
M6-B
AU
Minutes

Mutant + substrate Cylization @ 5μM + 50μM.

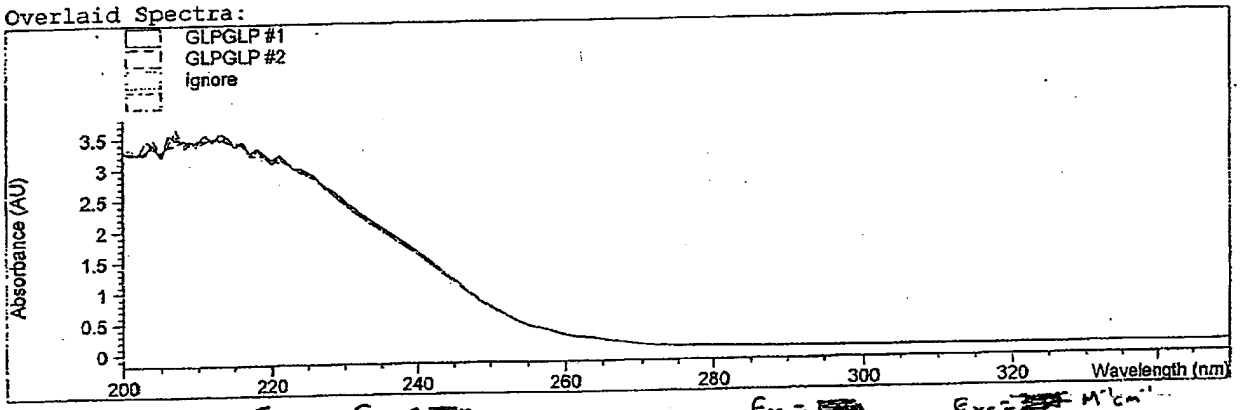
Mutant	Amt	$\epsilon_{280}^{(est)}$ ($M^{-1}cm^{-1}$)	MW	μL dissolved	A_{280}	# measurements	Conc
TLP-M1	1.2 mg	1280	1617.7	148 μL	.2331	2	4.55 mM
TLP-M2	1.0 mg	1280	1541.6	130 μL	.2331	2	4.55 mM
TLP-M3	1.0 mg	1280	1591.7	252 μL (w/5mM)	.1621	3	3.17 mM
TLP-M4	2.3 mg	1280	1575.6	272 μL	.2429	3	4.74 mM
TLP-M5	3.8 mg	1280	1470.5	517 μL	.1962	2	3.83 mM
TLP-M6	0.7 mg	1280	1688.8	166 μL (w/2.5mM)	.1075	2	2.10 mM
GLP-GLP	2.8 mg		1602.7	200 μL			mM 8.73

#	Name	Abs<280nm>	#	Name	Abs<280nm>
1	TLPM1 #1	0.23282	8	TLPM4 #1	0.23152
2	TLPM1 #2	0.23330	9	TLPM4 #2	0.24377
3	TLPM2 #1	0.22852	10	TLPM4 #3	0.25348
4	TLPM2 #2	0.23761	11	TLPM5 #1	0.19374
5	TLPM3 #1	0.15737	12	TLPM5 #2	0.19859
6	TLPM3 #2	0.16524	13	TLPM6 #1	0.10589
7	TLPM3 #3	0.16367	14	TLPM6 #2	0.10917

1.143

Spec of glpglp decapeptide 350 μM

Overlaid Spectra:



1:25 dilution For 1M: ϵ_{220} = 8846 ϵ_{250} = 2096 ϵ_{265} = 450 $M^{-1}cm^{-1}$

#	Name	Abs<220nm>	Abs<240nm>	Abs<250nm>	Abs<265nm>
1	GLPGLP #1	3.12700	1.62520	0.72974	0.16068
2	GLPGLP #2	3.09230	1.59180	0.71129	0.15603
3	~~ignore~~	~~3.07710~~	~~1.57870~~	~~0.70660~~	~~0.15546~~
4	GLPGLP #3	3.08120	1.57790	0.70647	0.15559

#	Name	Abs<280nm>	#	Name	Abs<280nm>
1	GLPGLP #1	3.6455E-2	~~3~~	~~ignore~~	~~3.5021E-2~~
2	GLPGLP #2	3.4674E-2	4		3.5275E-2

For cyclization rxns make up 10x stock of TLP + Mutants + GLPGLP.

	Stock	dilute stock → 500 μM	dilute 500 μM → 50 μM
TLP1	3.45 mM	17.4 μL → 120 μL	10 μL → 100 μL
TLP-M1	4.55 mM	13.2 μL → 120 μL	"
TLP-M2	4.55 mM	13.2 μL → 120 μL	"
TLP-M3	3.17 mM	18.9 μL → 120 μL	"
TLP-M4	4.74 mM	12.7 μL → 120 μL	"
TLP-M5	3.63 mM	15.7 μL → 120 μL	"
TLP-M6	2.10 mM	28.6 μL → 120 μL	"
GLPGLP	4.73 mM	6.9 μL → 120 μL	"

Make up dilution of TyrTE as per 1/21/00 to generate 80 mM stock.
Make one set for all 50 μM RXNS. and fresh for 5 μM RXNS

RXNS:

As per 1/21 for normal rxns + Ø time points.

Store samples @ -80°C.

Repeat cyclization of TLP1-SNac w/ 8 mM TyrTE.

TLP1 stock: $A_{280} = (1/2)(.2280 + .2369) = .2324$ for 1:25 dilution. = 4.54 mM

Dilutions:

500 μM	46.3 μL → 420 μL	
350 μM	126 μL → 180 μL	Dilute TyrTE as per 1/21/00 to give 80 mM stock. Make fresh for each set of rxns.
200 μM	40 μL → 100 μL	
150 μM	54 μL → 180 μL	
100 μM	60 μL → 300 μL	
60 μM	100 μL → 180 μL	Rxns:
35 μM	35 μL → 100 μL	Run a set with Ø time point at each
20 μM	20 μL → 100 μL	15 μM + 35 μM. Procedure as per 1/21/00.
10 μM	10 μL → 100 μL	

dilute from 500 μM

dilute from 100 μM

Final results for TycC TE catalyzed peptide-SNAC cyclization experiments

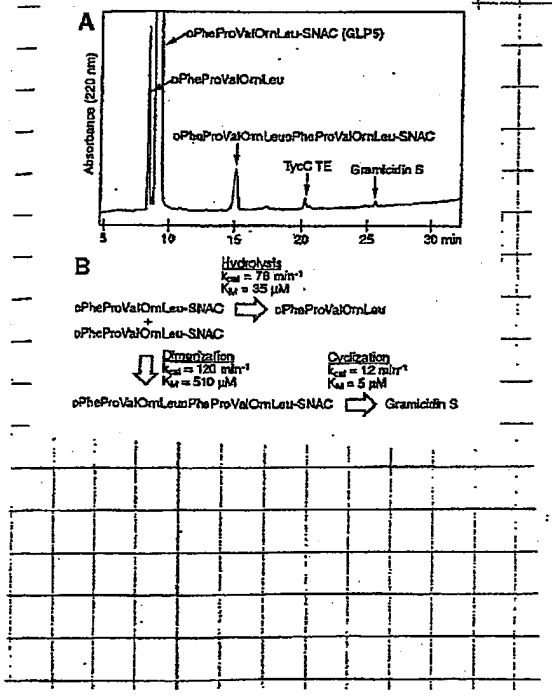
Table 1. Sequences of peptide-SNAC substrates, kinetics of TycC TE-catalyzed peptide-SNAC cyclization, and exact masses of cyclic peptide products (SNAC denotes N-acetylcysteamine thioester).

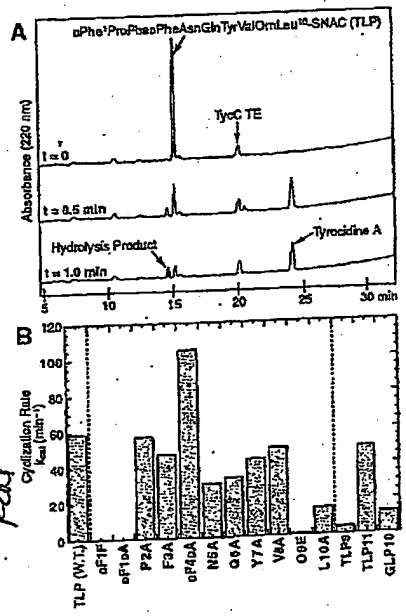
MALDI-TOF MS

		Cyclization			Cyclic Peptide	
Peptide-SNAC Substrate*		k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)	M+H (calculated)	M+H (observed)
TLP	DPheProPheDPheAsnGlnTyrValOrnLeu-SNAC	59 ± 13	3 ± 1	21	1270.7	1270.7
DF1F	PheProPheDPheAsnGlnTyrValOrnLeu-SNAC	< 0.05†	–	–	1270.7	not detected
DF1DA	DAlaProPheDPheAsnGlnTyrValOrnLeu-SNAC	< 0.05†	–	–	1194.6	not detected
P2A	DPheAlaPheDPheAsnGlnTyrValOrnLeu-SNAC	57	3	20	1244.6	1244.4
F3A	DPheProAlaDPheAsnGlnTyrValOrnLeu-SNAC	47	6	8	1194.6	1194.7
DF4DA	DPheProPheDAlaAsnGlnTyrValOrnLeu-SNAC	105	6	16	1194.6	1194.7
N5A	DPheProPheDPheAlaGlnTyrValOrnLeu-SNAC	30	6	5	1227.7	1227.8
Q6A	DPheProPheDPheAsnAlaTyrValOrnLeu-SNAC	33	4	8	1213.6	1213.7
Y7A	DPheProPheDPheAsnGlnAlaOrnLeu-SNAC	43	15	3	1178.4	1178.8
V8A	DPheProPheDPheAsnGlnTyrAlaOrnLeu-SNAC	49	9	5	1242.6	1242.6
O9E	DPheProPheDPheAsnGlnTyrValGluLeu-SNAC	0.5	56	0.01	1285.6	1285.6
L10A	DPheProPheDPheAsnGlnTyrValOrnAla-SNAC	15	6	3	1228.6	1228.7
TLP9	DPheProPheAsnGlnTyrValOrnLeu-SNAC	4	6	0.6	1123.6	1123.8
TLP11	DPheProPheDPheAsnAlaGlnTyrValOrnLeu-SNAC	49	20	2	1341.7	1341.4
GLP10	DPheProValOrnLeuDPheProValOrnLeu-SNAC	12	5	2	1141.7	1141.8

*Residues that differ from those in the wild-type substrate **TLP** are in bold type. †Lower limit of detection.

Many of the Fokefic assays done by Rahul Kohli





kcat

Cyclization reactions: look for cyclization

Stock solutions: (3-5 mM)

3 mM ① FLP1 (MW = 1484.6, TFA salt), 2.2 mg → 3 mM
dissolve in 494 λ H_2O ⇒ 3 mM

2.4 mM ② TL03

2.43 mM stock solution previously prepared

3 mM ③ M17 (MW = 354.4, 2 TFA salt), 3.5 mg → 3 mM
dissolve in 862 λ H_2O ⇒ 3 mM

5 mM ④ M18 (MW = 1109.1, 2 TFA salt), 3.2 mg → 3 mM
dissolve in 962 λ H_2O ⇒ 3 mM

1 mM ⑤ Phelac (MW = 504.7, 1 TFA salt), 1.4 mg → 1 mM
dissolve in 930 λ ⇒ 1 mM

Enzymes:

	TE	prep	conc
(A)	TycCTE	TE 89	40 μM
(B)	PCP1DTE	PCP1078	23 μM
(C)	FenTE	FenTE 46	97 μM

substrate) 2 μM 10 μM 50 μM

enzyme: ~~500 nM~~ 200 nM TE

Cyclization conditions:

30 - 200 μM peptide-SNAC
200 mM ~~Tris~~ TE
25 mM MOPS, pH 7.0
} total volume of 400 μL

time = 2 min.

Enzyme { PCP1OTE x 1
FenTE x 1
TycC TE x 4

Reactions

Rxn	TE		Substrate		Amt. Peptide stock		Amt H2O
1	TycC TE	200 μM	TLP3	(320)	33 λ		287 λ
2	TycC TE	200 μM	PheOacl	(320)	80 λ		240 λ
3	TycC TE	180 μM	M17	400	27 λ		373 λ
4	TycC TE	180 μM	M18	400	27 λ		373 λ
5	~~Eggs~~ PCP1OTE	200 μM	TLP3	(320)	33 λ	160 μM	287 λ
6	FenTE	180 μM	FLP1	400	27 λ		373 λ

Enzyme stocks:

10.6 λ 40 μM TycC TE
189 λ ~~H2O~~ TE buff
200 λ 2 μM TycC TE

5 ~~4~~ λ 23 μM PCP1OTE
45 ~~45~~ λ ~~H2O~~ TE buff
50 λ PCP1OTE, 2 μM

4.3 λ 92 μM FenTE
186 λ ~~H2O~~ TE buff
200 ~~400~~ λ, 2 μM FenTE

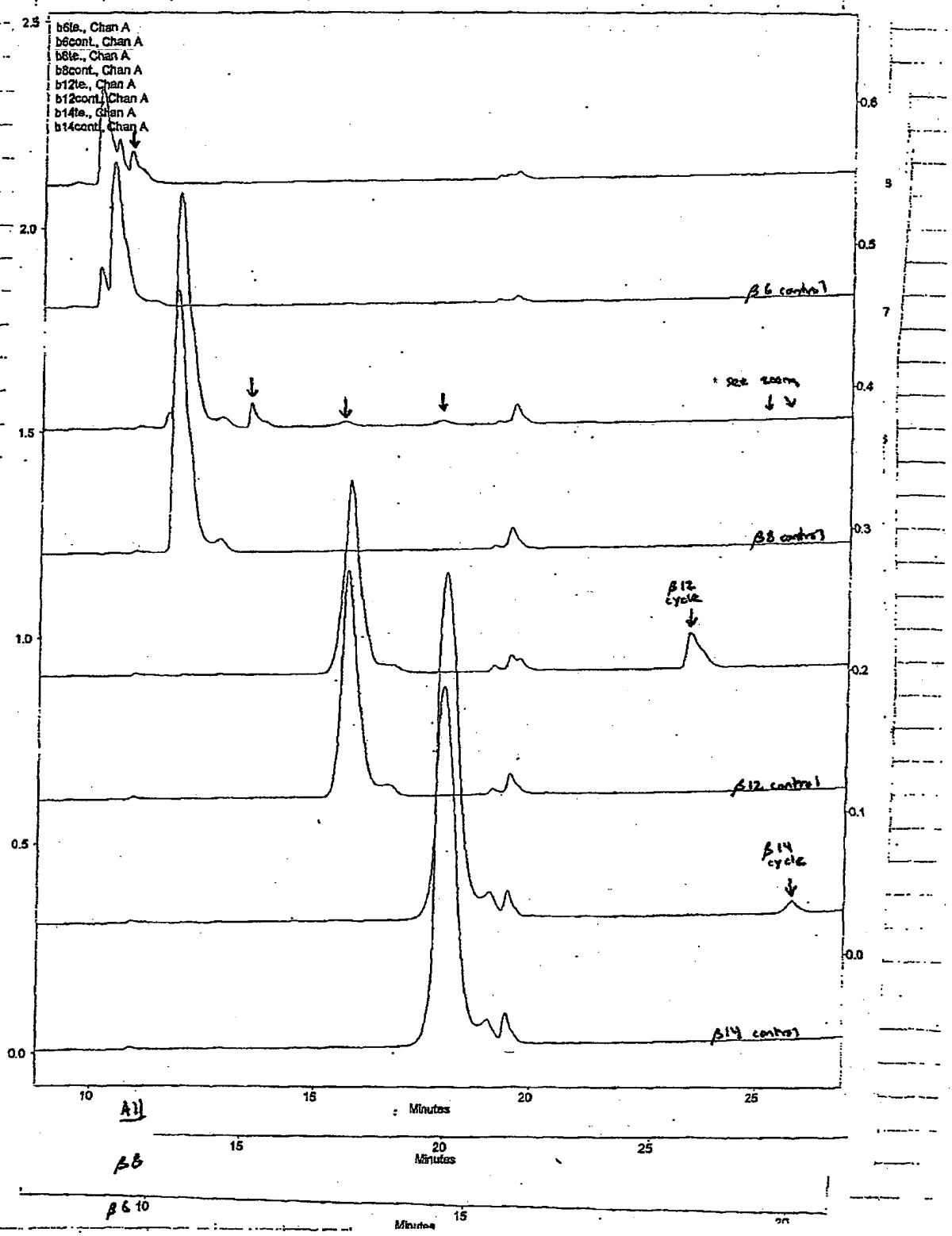
TE buff = 10 mM MOPS, pH 7,
10 mM NaCl

Reaction

40 λ 250 mM MOPS, pH 7
λ or 320 λ H2O + peptide - SNAC
40 λ TE stock
~~400~~ λ
2 min at RT
- then + 25 λ 17% TFA/water quench
- Freeze LN2
- +80 λ CH3CN (17% F/V)

↓ = new products

HPLC of Samples.



All

β8

β6 10

15

20

25

Minutes

Reaction of β-series w/ TycC TE.

Stock soln of β-series peptides.

	Amt	MW	DDW	[Final]
β6	2.1 mg	1209.4	[redacted] 347 μL	5 mM
β8-1	1.6 mg	1403.6	[redacted] 456 μL	2.5 mM
β12	2.0 mg	1827.9	438 μL	2.5 mM
β14	1.6 mg	2026.0	790 μL	1 mM

↳ purity estimated @ 50%, thus true final [M] near 500 μM

Rxn:

Make 500 μM stock of each peptide-SNAC.

β6	dilute	10 μL →	100 μL
β8	dilute	20 μL →	100 μL
stock = 8.73 mM ← glp10	dilute	5.7 μL →	100 μL
β12	dilute	20 μL →	100 μL
β14	use	stock. (purity 50% →	~500 μM)

→ 22.6 μL 20 μM + 426.4 μL DDW } 450 μL 2 μM TycTE.

Rxn	(500 μM) peptide	250 mM MOPS, pH 7	DDW	+ 2 μM TycTE to initiate	
β6 [redacted] TE	40 μL	40 μL	280 μL	40 μL	→ 1' – add 25 μL 1.7% TFA Flash freeze
β6 [redacted] cont	"	"	"	—	
β8 [redacted] TE	"	"	"	40 μL	→ 1' add 25 μL 1.7% TFA Flash freeze
β8 [redacted] cont	"	"	"	—	
g10 [redacted] TE	"	"	"	40 μL	→ 1' – add 25 μL 1.7% TFA FF
g10 [redacted] cont	"	"	"	—	
β12 [redacted] TE	"	"	"	40 μL	→ 1' – add 25 μL 1.7% TFA FF
β12 [redacted] cont	"	"	"	—	
β14 [redacted] TE	"	"	"	40 μL	→ 1' – add 25 μL 1.7% TFA FF
β14 [redacted] cont	"	"	"	—	

For all cont rxn. add first 25 μL 1.7% TFA.
then add 40 μL 2 μM TycC TE
Flash freeze instantly.